与が推奨されている．ステロイドの中止時期については残念ながら明確な解答が得られていない．また，心サ症においてステロイド療法からメトトレキサート療法に変更してメトトレキサートの有効性が認められなかった報告[34]やプレドニゾロンとメトトレキサート併用が有効であった報告[35]がある．

心サ症の治療においては，ステロイド療法に加えて，高度房室ブロックなど徐脈性不整脈に対する恒久的ペースメーカー植え込み術，重症心室性不整脈に対する抗不整脈薬，電気的焼灼術（カテーテルアブレーション），植え込み型除細動器（ICD）が必要な場合がある．さらに慢性心不全に対しては薬物療法（ジギタリス，利尿薬，アンジオテンシン変換酵素阻害薬，アンジオテンシン受容体拮抗薬，β遮断薬など）が行われる．最近，慢性心不全に対する非薬物療法の一つに両心室ペーシング治療が注目されているが，心サ症に同療法を施行し心機能が改善したとの報告がある[36, 37]．心サ症の患者では既に右室に恒久的ペースメーカーが植え込まれている場合も多く，このような症例で慢性心不全が出現した時には両心室ペーシング治療の良い適応になる場合がある．

サ症治療における最近のトピックスとしてHMG-CoA還元酵素阻害薬（statin）に関するものがあり，atorvastatinがTh1/Th2バランスを調節して，いわゆるTh1病の治療に有効である可能性が報告されている[38]．一方pravastatinがサ症に合併したミオトニアを増悪させたとする報告もある[39]．また，*Propionibacterium acnes*など細菌がサ症の起因体である可能性[40]から皮膚サ症などに対してエリスロマイシン系などの抗生物質による治療が試みられているが[41]現在まで心サ症に関する報告はない．

結論

心サ症の診断は心臓以外の臓器病変が明かでない場合，しばしば拡張型心筋症または原因不明の心筋症と誤診されている．また，本症の疫学，病因，病態（活動性），治療，予後などについても未だ不明な点が多く，感度および特異性が高い診断法，的確な活動性の評価法の確立のみならず病因・病態の解明が待たれる．しかし，幸いにサ症の病因・病態の解明が急速に進行しており，近い将来これらが解決されることが期待できる．

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Apoptotic myocardial cell death in the setting of arrhythmogenic right ventricular cardiomyopathy

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Background — Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a primary heart muscle disease characterized by progressive atrophy of the right ventricular myocardium accompanied by fibro-fatty replacement. We examined whether the loss of myocardial cells in the setting of ARVC could result from cell death by apoptosis as demonstrated by the detection of DNA fragmentation and the expression of apoptosis regulatory proteins (e.g. CPP-32, Bcl-2, and Bax).

Methods — Specimens obtained from the right ventricular myocardium of 11 patients with ARVC (ARVC group) and 10 age-matched normal individuals (control group) were analysed. To identify individual cells undergoing apoptosis, paraffin sections were examined with the TdT-mediated dUTP-biotin nick end labelling method (TUNEL) and the rabbit polyclonal anti-single stranded DNA method (ssDNA). The apoptotic index was calculated as the percentage of nuclei staining positive by the TUNEL or ssDNA method. We also used immunohistochemical techniques to examine the levels of CPP-32, Bcl-2, and Bax expression.

Results — Apoptosis was detected in the ARVC group with a mean apoptotic index of 5.7 ± 4.5% (ssDNA) and 23.8 ± 7.5% (TUNEL), but not in the control group ($p < 0.01$). CPP-32 expression and Bax overexpression were observed in the ARVC group. However, Bcl-2 expression was not seen in either group.

Conclusions — Apoptotic myocardial cell death occurs in the setting of ARVC and may contribute to the loss of myocardial cells. (Acta Cardiol 2005; 60(5): 465-470)

Keywords: *arrhythmia – cardiomyopathy – apoptosis – pathology.*

Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a primary heart muscle disorder of unknown cause and is characterized pathologically by progressive loss of myocardium with fibro-fatty replacement[1]. The affected area is the free wall of the right ventricle while the interventricular septum is usually spared. A variable amount of left ventricular involvement has also been identified in some patients[1,2].

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Apoptosis (programmed cell death) is a genetically mediated process that allows individual cells to be deleted from tissues[3]. Apoptosis can be detected by two recently developed *in situ* techniques, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling method (TUNEL)[4] and the rabbit polyclonal anti-single stranded DNA method (ssDNA)[5], was introduced to identify apoptotic cells in paraffin-embedded tissue sections. The former method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the 3'-OH ends of DNA breaks that can be identified by immunohistochemical detection of biotin-labelled dUTP. The latter method is based on denaturation of DNA *in situ* in the presence of Mg^{2+} with subsequent staining with an antibody specific for single-stranded DNA.

Apoptosis has been postulated to account for progressive myocardial atrophy, which is a typical feature of ARVC and a cause of cardiac electrical instability[1,6]. Evidence of apoptosis in the setting of ARVC has been documented in postmortem specimens[7], but whether postmortem autolytic cell death may also cause cell nuclei to be TUNEL positive remains controversial[8]. To identify individual cells undergoing apoptosis, paraffin sections obtained from the right ventricular myocardium of 11 patients with ARVC were examined with the TUNEL and ssDNA methods. We also used immunohistochemical techniques to determine the levels of CPP-32, Bcl-2, and Bax expression in relation to the degree of apoptosis.

Methods

The diagnosis of ARVC was established in 11 patients based on the standardized criteria of McKenna et al.[9], which encompass structural, histological, electrocardiographic, arrhythmic, and genetic factors. The clinical diagnosis of ARVC was fulfilled by the presence of two major criteria, one major plus two minor criteria, or four minor criteria. The histomorphometry of endomyocardial biopsy specimens was determined according to Angelini et al.[10]. Specifically, the presence of fatty tissue and fibrous tissue exceeding 3.21% and 40.38%, respectively, should be considered highly suggestive of arrhythmogenic right ventricular cardiomyopathy in right ventricular endomyocardial biopsy samples.

Eight patients were men and three were women (59.2 ± 17.2 years), and specimens were obtained at the time of cryosurgery. Samples from 10 age-matched normal individuals (3 men and 7 women, 54.2 ± 19.2 years) were analysed as controls. The control samples were specimens of right ventricles obtained from autopsied hearts in patients without apparent cardiac diseases. The samples were fixed for 3 to 4 h in 10% phosphate-buffered formalin, 3-μm paraffin-embedded sections were cut and stained according to the haematoxylin and eosin and Masson's trichrome techniques.

TUNEL

The samples were fixed for 3 to 4 h in 10% phosphate-buffered formalin, 3-μm paraffin-embedded sections were cut, and mounted on glass slides pretreated with saline. Tissue sections were deparaffinized, hydrated by transferring the slides through the following solutions: twice in a xylene bath for 5 min, and then for 2 min in 100% ethanol, and then washed with distilled water. After this procedure, we were careful not to allow the tissue sections to dry. Then the slides were incubated with 20 μg of proteinase K per milliliter of phosphate-buffered saline. Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxide. Tissue sections were stained using the MEBSTAIN Apoptosis Kit II system (Medical & Biological Laboratories, Nagoya, Japan) that identifies cells with internucleosomal DNA fragmentation of apoptosis. The procedure was performed according to the manufacturer's instructions. The method is based on the preferential binding of terminal deoxynucleotidyl transferase (TdT) to the 3'-hydroxyl ends of DNA[4]. Briefly, residues of biotinylated deoxyuridine triphosphate (dUTP) were catalytically added to the ends of DNA fragments by the enzyme TdT. After end-labelling, the sections were incubated with avidin-conjugated FITC to detect the biotin-labelled nuclei. Apoptotic bodies were identified by fluorescent microscopy. Human thymus tissue was used as a positive control. Sections were first examined under fluorescent microscopy at low magnification (× 160), and at least 100 myocytes were counted. The apoptotic index was calculated as the number of positively staining myocytes divided by total myocyte number and multiplying that value by 100. Cardiomyocytes were easily distinguished morphologically from other rare nonmyocytes under fluorescent microscopy at high magnification (× 320).

Immunohistochemical detection of polyclonal rabbit anti-single stranded DNA

Polyclonal rabbit anti-single stranded DNA (ssDNA) recognizes DNA fragmentation caused by single-strand breaks in nuclei during programmed cell death, because this antibody binds specifically to ssDNA that is more than 5 or 6 nucleotides in length and does not cross-react with double-stranded DNA or RNA. Reactivity has been observed in apoptotic lymphocytes in the germinal centre of lymphoid follicles as well as in tumour cells. In addition, the nuclei of drug-induced apoptotic cells and cells that have undergone programmed cell death in the mouse embryo react with this antibody[11].

After deparaffinization and hydration, endogenous peroxidase was inactivated by the addition of 3% hydrogen peroxide. All sections were incubated with polyclonal rabbit anti-ssDNA antibody (1:200 dilution, DAKO JAPAN co., Ltd., Kyoto, Japan) for 60 min at room temperature, washed 3 times for 5 min in phosphate-buffered saline and incubated with EnVision+™ peroxidase-conjugated anti-rabbit antibody (DAKO co., Ltd., Carpinteria, CA, USA) for 30 min at room temperature. Chromogenic identification of bound antibody was performed. Human thymus was used as a control.

Immunohistochemical detection of CPP-32 protease

CPP-32 is a cysteine protease required for the initiation of apoptotic cell death[12]. It is related to interleukin-1β,-converting enzyme (ICE) and CED-3, the product of a gene required for programmed cell death in the nematode *Caenorhabditis elegans*. CPP-32 is the specific ICE/ CED-3-like mammalian cysteine protease that cleaves and inactivates poly (adenosine diphosphate ribose) polymerase, an enzyme involved in DNA repair and genome integrity, and thus may be the human equivalent of CED-3[12]. Therefore, to provide further evidence for the presence of apoptosis in the setting of arrhythmogenic right ventricular cardiomyopathy, we determined the level of expression of CPP-32 in the right ventricles of the patients and control individuals using immunohistochemical techniques.

After deparaffinization and hydration, endogenous peroxidase was inactivated by the addition of 3% hydrogen peroxide. All sections were incubated with polyclonal rabbit anti-human CPP-32 (1:250 dilution, DAKO Co., Ltd., Carpinteria, CA, USA) for 60 min at room temperature, washed 3 times for 5 min in phosphate-buffered saline and incubated with EnVision+™ peroxidase-conjugated anti-rabbit antibody (DAKO) for 30 min at room temperature. Chromogenic identification of bound antibody was performed. Human thymus was used as a control.

Immunohistochemical detection of Bax and Bcl-2

The protein encoded by the bcl-2 gene is a regulator of programmed cell death and apoptosis. The cell survival-promoting activity of this protein is opposed by Bax, a homologous protein that forms heterodimers with Bcl-2 and accelerates rates of cell death[13]. Bax homodimerizes and forms heterodimers with Bcl-2 *in vivo*. Overexpressed Bax accelerates apoptotic cell death induced by cytokine deprivation in an IL-3-dependent cell line and also antagonizes the death repressor activity of Bcl-2. Therefore, it has been suggested that the ratio of Bcl-2 to Bax determines survival or death following exposure to an apoptotic stimulus[14].

After deparaffinization and hydration, endogenous peroxidase was inactivated by the addition of 3% hydrogen peroxide. All sections were incubated with polyclonal rabbit anti-human Bax antibody (1:1500 dilution, DAKO Co., Ltd., Carpinteria, CA, USA) for 60 min at room temperature, washed 3 times for 5 min in phosphate-buffered saline and incubated with EnVision+™ peroxidase-conjugated anti-rabbit antibody (DAKO) for 30 min at room temperature. Chromogenic identification of bound antibody was performed. Human thymus was used as a control.

On the other hand, after deparaffinization and hydration, endogenous peroxidase was inactivated by the addition of 3% hydrogen peroxide. All sections were incubated with monoclonal mouse anti-human Bcl-2 antibody (1:1500 dilution, DAKO Co., Ltd., Carpinteria, CA, USA) for 60 min at room temperature, washed 3 times for 5 min in phosphate-buffered saline and incubated with EnVision+™ peroxidase-conjugated anti-mouse antibody (DAKO) for 30 min at room temperature. Chromogenic identification of bound antibody was performed. Human thymus was used as a control.

Statistical analysis

All data are presented as the mean ± standard deviation. Significant differences were determined using Student's t test (StatView-J 4.11). A value for $P < 0.05$ was considered significant.

Results

The nuclei of the TUNEL-positive cells are identified by green under fluorescent microscopy because avidin-conjugated FITC was used for specific staining (Fig. 1A). In contrast, the nuclei of the ssDNA-positive cells are stained brown (Fig. 1B). The cytoplasm of the CPP-32-positive cells is stained brown (Fig. 2). The cytoplasm of the Bax-positive cells is also stained brown (Fig. 3A), but Bcl-2-positive cells are not identified (Fig. 3B).

The results of the ARVC group are summarized in Table 1. The apoptotic index by the TUNEL method (23.7 ± 7.6%) is much greater than that by the ssDNA method (5.7 ± 4.5%). All of the specimens in the ARVC group were stained with the CPP-32 antibody and Bax antibody, but did not stain with the Bcl-2 antibody.

The apoptotic indexes of the control group were 10.2 ± 8.3% by the TUNEL method and 0% by the ssDNA method ($p < 0.01$), which are not shown in the table. None of the control specimens stained with the CPP-32 antibody, the Bax antibody, or the Bcl-2 antibody.

Discussion

The TUNEL method and the ssDNA method

The TdT-mediated dUTP-biotin nick end labelling method (TUNEL method) developed by Gavrieli et al.[4] has enabled *in situ* visualization of DNA fragmentation at the single cell level and is considered to be a more sensitive method than conventional morphological techniques. Therefore, the detection of apoptotic cells is possible in a variety of histological specimens. However,

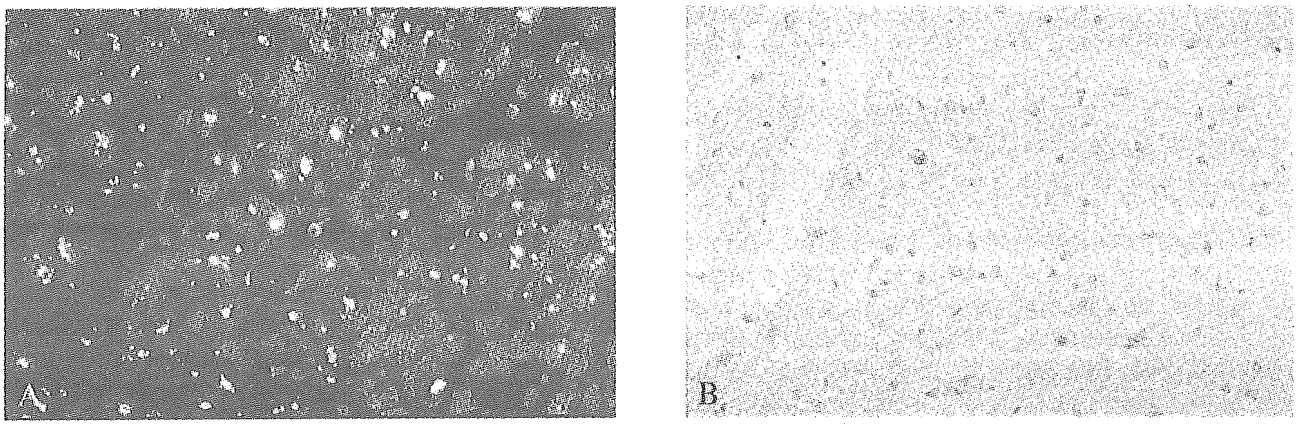

Fig. 1. – The nuclei of the TUNEL-positive cells are identified by green under fluorescent microscopy because avidin-conjugated FITC was used for specific staining at a magnification of × 320 (A). In contrast, the nuclei of the ssDNA-positive cells are stained brown at a magnification of × 400 (B).

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Fig. 2. – The cytoplasm of the CPP-32-positive cells is stained brown at a magnification of × 400.

Grasl-Kraupp et al.[9] reported that the TUNEL reaction can occur in necrotic cells and in insufficiently fixed autolytic cells because the TUNEL method detects DNA fragmentation. Because the TUNEL method is complicated, it is possible that non-specific reactions are amplified.

For the ssDNA method, we used the polyclonal rabbit anti-single stranded DNA (ssDNA) antibody. The ssDNA antibody specifically recognizes DNA fragmentation of more than 5 or 6 bases caused from a single strand break during programmed cell death, but it does not cross-react with double-stranded DNA or RNA. Therefore, the specificity of the ssDNA method is much greater than that of the TUNEL method[5].

In this study, because the TUNEL method was used for specimens that were fixed in 10% phosphate-buffered formalin, we hypothesize that non-specific reactions occurred in the cells. Therefore, the apoptotic index based on the TUNEL method (23.7 ± 7.6%) is much greater than that based on the ssDNA method (5.7 ± 4.5%). Therefore, the ssDNA method is thought to be much easier to perform and more specific than the TUNEL method for the detection of apoptotic cells.

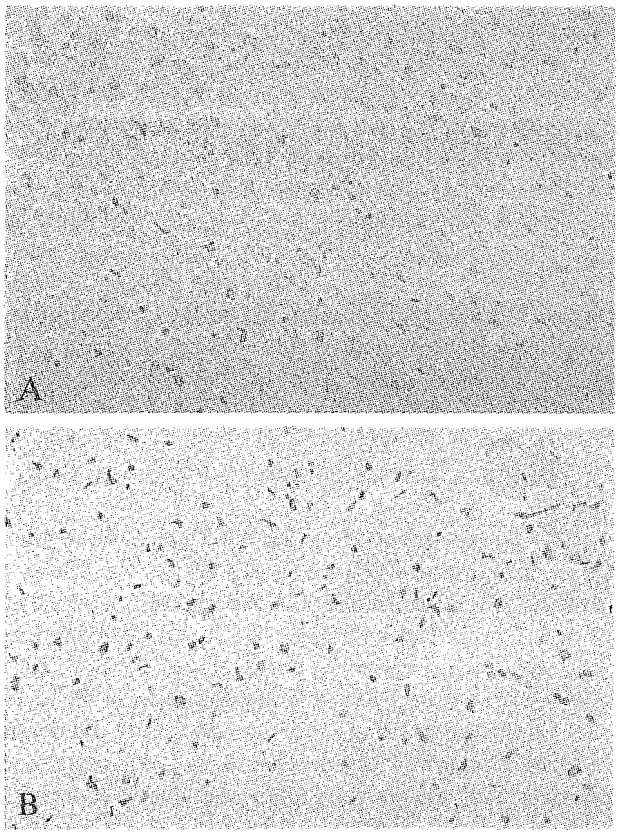

Fig. 3. – The cytoplasm of the Bax-positive cells is stained brown at a magnification of × 400 (A), but Bcl-2-positive cells are not identified at a magnification of × 400 (B).

The detection of the apoptotic cells by the CPP-32 antibody

CPP-32 is a putative mammalian cysteine protease that is responsible for the cleavage and inactivation of poly (ADP-ribose) polymerase, which appears to be involved in DNA repair or genome surveillance and integrity, and is necessary for apoptosis. This proenzyme is related to interleukin-1β,-converting enzyme (ICE) and CED-3, the product of a gene required for

Table 1. - *Summarized results of the ARVC group*

Patient number	Age (years)/gender	TUNEL (%)	ssDNA (%)	CPP-32	Bax/Bcl-2
1	41/M	19.9	2.0	positive	positive/negative
2	18/M	26.2	4.0	positive	positive/negative
3	52/M	19.9	4.9	positive	positive/negative
4	53/F	22.5	5.1	positive	positive/negative
5	33/M	9.5	2.0	positive	positive/negative
6	55/M	20.5	12.7	positive	positive/negative
7	56/F	22.5	6.7	positive	positive/negative
8	46/M	22.2	3.5	positive	positive/negative
9	55/M	25.6	15.8	positive	positive/negative
10	42/F	35.9	3.0	positive	positive/negative
11	46/M	41.8	3.0	positive	positive/negative
		23.7 ± 7.6	5.7 ± 4.5		

programmed cell death in *Caenorhabditis elegans*. A potent peptide aldehyde inhibitor has been developed and shown to prevent apoptotic events *in vitro*, suggesting that CPP-32 is important for the initiation of apoptotic cell death[12]. In all of the specimens from the ARVC group, we identified definite CPP-32 expression, but not in the control group. Therefore, in the ARVC group, the loss of cardiomyocytes may be the result of apoptosis.

Bax and Bcl-2

The protein encoded by the bcl-2 gene is a regulator of programmed cell death and apoptosis. The cell survival-promoting activity of this protein is opposed by Bax, a homologous protein that forms heterodimers with Bcl-2 and accelerates rates of cell death. Bax forms homodimers and heterodimers with Bcl-2 *in vivo*. When Bax expression predominates, programmed cell death is accelerated, and the death repressor activity of Bcl-2 is inhibited. Therefore, it is believed that the ratio of Bcl-2 to Bax expression determines the susceptibility to cell death following an apoptotic stimulus[13,14]. When Bcl-2 is in excess, cells are protected, but when Bax is in excess and Bax homodimers dominate, cells are susceptible to apoptosis.

In all of the samples in the ARVC group, we noted Bax overexpression, but not Bcl-2 expression. In the control group, we were not able to identify Bax expression or Bcl-2 expression. Therefore, in the ARVC group, the loss of cardiomyocytes may have been the result of apoptosis.

In conclusion, apoptotic myocardial cell death occurs in ARVC and may contribute to the loss of myocardial cells that is consequently thought to result in heart failure.

Study limitations

We have analysed a relatively small number of heart specimens from patients with ARVC. For the detection of apoptotic cardiomyocytes, we did not assess DNA laddering or detect apoptotic bodies by electron microscopy. However, we were still able to show differences in the rate of apoptosis between the ARVC group and the control group using immunohistochemical techniques.

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